Agrin Is a Major Heparan Sulfate Proteoglycan Accumulating in Alzheimer's Disease Brain

Marcel M. Verbeek,* Irene Otte-Höller,*
Jacob van den Born,[†]
Lambert P. W. J. van den Heuvel,[‡] Guido David,[§]
Pieter Wesseling,*[¶] and Robert M. W. de Waal*

From the Departments of Pathology,* Nephrology,[†] Pediatrics,[‡]
and Neurology,[§] University Hospital Nijmegen, Nijmegen, The
Netherlands; and the Center for Human Genetics,[§] University of
Leuven and Flanders Interuniversity Institute for Biotechnology,
Leuven, Belgium

Heparan sulfate proteoglycans (HSPGs) have been suggested to play an important role in the formation and persistence of senile plaques and neurofibrillary tangles in dementia of the Alzheimer's type (DAT). We performed a comparative immunohistochemical analysis of the expression of the HSPGs agrin, perlecan, glypican-1, and syndecans 1–3 in the lesions of DAT brain neocortex and hippocampus. Using a panel of specific antibodies directed against the protein backbone of the various HSPG species and against the glycosaminoglycan (GAG) side-chains, we demonstrated the following. The basement membrane-associated HSPG, agrin, is widely expressed in senile plaques, neurofibrillary tangles and cerebral blood vessels, whereas the expression of the other basement membrane-associated HSPG, perlecan, is lacking in senile plaques and neurofibrillary tangles and is restricted to the cerebral vasculature. Glypican and three different syndecans, all cell membrane-associated HSPG species, are also expressed in senile plaques and neurofibrillary tangles, albeit at a lower frequency than agrin. Heparan sulfate GAG side chains are also associated with both senile plaques and neurofibrillary tangles. Our results suggest that glycosaminoglycan side chains of the HSPGs agrin, syndecan, and glypican, but not perlecan, may play an important role in the formation of both senile plaques and neurofibrillary tangles. In addition, we speculate that agrin, because it contains nine protease-inhibiting domains, may protect the protein aggregates in senile plaques and neurofibrillary tangles against extracellular proteolytic degradation, leading to the persistence of these deposits. (*Am J Pathol* 1999, 155:2115–2125)

The brains of patients with dementia of the Alzheimer's type (DAT) are characterized by extensive formation of

neurofibrillary tangles, senile plaques, and vascular amyloid angiopathy.¹ Tangles primarily consist of fibrillar aggregates of hyperphosphorylated tau protein.^{2,3} The amyloid β protein ($A\beta$) is the major component of both senile plaques and cerebrovascular amyloid angiopathy.⁴ Besides these fibril-forming constituents, a number of $A\beta$ -associated and tau protein-associated molecules have been identified in tangles and senile plaques, among which are inflammatory proteins, apolipoproteins, and proteoglycans.^{5,6}

Heparan sulfate proteoglycans (HSPGs) are an invariable component of all kinds of systemic amyloids that occur in humans.⁷ With antibodies directed either against the glycosaminoglycan (GAG) side chains or the protein core of basement membrane-derived HSPG,⁸ HSPGs have been identified in tangles, senile plaques, and in cerebrovascular amyloid angiopathy in DAT patients.^{9–13} Furthermore, HSPGs have been identified by indirect methods, eg, basic fibroblast growth factor binding.^{14,15} At that time, the specific protein sequences of the HSPGs were unknown. Later, the first basement membrane-derived HSPG was cloned and named perlecan.¹⁶ It was reported that perlecan was expressed in senile plaques of the cortex, but not of the cerebellum, that usually do not transform into fibril-containing classic senile plaques.¹⁷ Because perlecan can bind to both $A\beta$ and the amyloid precursor protein (β PP) through its core protein or GAG moieties,^{18–21} this molecule may play a significant role in amyloid formation in Alzheimer's disease, eg, by affecting the processing of β PP or by determining the localization of $A\beta$ deposition at sites where perlecan is produced. This suggested that HSPGs may be involved in the transformation of $A\beta$ into fibrils, which has been confirmed *in vitro* and in rats.^{18,22} This activity may be predominantly mediated by the sulfate moieties of GAGs.^{23–25} Furthermore, heparan sulfate also increases serum amyloid A₂ fibril formation.²⁶

HSPGs may also participate in the formation of tangles. Sulfated GAGs stimulate the phosphorylation of tau and inhibit the binding of tau to microtubules, thus promoting the formation of paired helical filaments.^{27–30} In addition, glycosylation of tau itself is important for the maintenance of paired helical filament structures.³¹

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Address reprint requests to Dr. Marcel M. Verbeek, 319 Lab Pediatrics & Neurology, University Hospital Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands. E-mail: m.verbeek@ckslkn.azn.nl.

Table 1. Antibodies Used

Primary antibody	Antigen	Species in which raised	Source* (reference)	Dilution	Preincubation serum (20%)	Secondary antibodies [†]
6C6	A β	Mouse	Athena (62)	5 μ g/ml	Horse	ABC
tau-2	Tau	Mouse	Sigma	1:800	Horse	ABC
AT-8	Tau	Mouse	Innogenetics	1:200	Horse	ABC
anti-tau [‡]	Tau	Rabbit	Sigma	1:100	Swine	SwaR/PAP
Q1510	Ubiquitin	Mouse	Chemicon	1:4000	Horse	ABC
JM-72	Agrin	Mouse	(38, 63)	1:500	Horse	ABC
BL-31	Agrin	Goat	(64)	1:500	Horse	DoGoPO
1948	Perlecan [§]	Rat	Chemicon	1:500	Rabbit	RaRaBio/ABC
HK-102	Perlecan [¶]	Rat	Seikagaku (40)	20 μ g/ml	Rabbit	RaRaBio/ABC
EY-90	Perlecan [§]	Rabbit	(8)	1:800	Swine	SwaR/PAP
95J10	Perlecan	Mouse	(41)	1:500	Horse	ABC
10H4	Syndecan-2	Mouse	(43, 65)	1:400	Horse	ABC
2E9	Syndecan-1, -3	Mouse	(42, 43)	1:500	Horse	ABC
1C7	Syndecan-3	Mouse	(42, 43)	1:50	Horse	ABC
S1	Glypican-1	Mouse	(44, 45)	1:50	Horse	ABC
3G10	HS stub	Mouse	(46)	1:250	Horse	ABC
JM-403	HS	Mouse (IgM)	(47, 66)	1:300	Goat	GoaM(IgM)PO
JM-13	HS	Mouse (IgM)	(67)	1:100	Goat	GoaM(IgM)PO
10E4	HS	Mouse (IgM)	Seikagaku (46)	1:100	Goat	GoaM(IgM)PO
ApoE	ApoE	Mouse	Innogenetics	1:400	Horse	ABC

*Commercial suppliers: Athena Neurosciences, San Francisco, CA; Sigma, St. Louis, MO; Innogenetics, Antwerpen, Belgium; Seikagaku Corporation, Tokyo, Japan; Chemicon, Harrow, England.

[†]ABC, avidin-biotin-peroxidase complex (Vector, Burlingame, CA); SwaR/PAP, swine anti-rabbit/PO-anti-PO (Dako, Glostrup, Denmark); DoGoPO, PO-labeled donkey anti-goat (Dako); RaRaBio, biotin-labeled rabbit anti-rat (Vector); GoaM(IgM)PO, PO-labeled goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL).

[‡]Applied only in double immunostaining.

[§]Directed against mouse perlecan, cross-reactive with human.

[¶]Directed against mouse perlecan, not cross-reactive with human.

^{||}Raised against human recombinant perlecan.

Most studies have focused on the role of the HSPG perlecan in the pathogenesis of DAT. Recently, however, a second basement membrane-derived HSPG was cloned, named agrin.^{32–34} Furthermore, various cell membrane-associated HSPGs have been described, that contain, among others, syndecan and glypican proteoglycans.³⁵ So far the differential expression of these types of HSPGs in DAT brains has hardly been studied.³⁶ To get more insight into the possible involvement of these proteoglycans in the pathogenesis of senile plaques and tangles, we studied, by immunohistochemistry of DAT and control brains, the expression of perlecan, agrin, syndecan 1–3, glypican-1, and HS GAG side-chains by using a panel of well-defined antibodies.

Materials and Methods

Tissue Samples

Brain tissue from patients with clinically diagnosed and neuropathologically confirmed DAT and from nondemented controls was obtained at autopsy. A definite diagnosis of DAT was based on a combination of neuropathological and clinical criteria.³⁷ Tissue samples from frontal and temporal neocortex and from the hippocampus were obtained from 11 DAT patients (6 females and 5 males; age 77 ± 9.4 years; postmortem delay 4.2 ± 1.2 hours) and 3 nondemented controls (3 females; age 80 ± 7.8 years; postmortem delay 5.3 ± 1.2 hours). Tissue samples were snap-frozen in liquid nitrogen immediately after removal.

Antibodies and Immunohistochemical Staining

The antibodies used in this study, their source, and dilutions are listed in Table 1. Details on the specific epitope that is recognized by the anti-HSPG monoclonal antibodies (mAbs) are as follows. mAb JM72 is directed against a peptide domain within agrin that encompasses several follistatin-like modules, a serine/threonine-rich module, and laminin-III-like epidermal growth factor (EGF) repeats.³⁸ mAb 1948 is directed against perlecan domain IV.³⁹ HK102 is directed against the protein core of mouse perlecan.⁴⁰ mAb 95J10 is directed against an epitope within amino acids 24–404 of perlecan (domains I and IIa).⁴¹ mAb 2E9 reacts with a peptide epitope common to the cytoplasmic domain of both syndecan-1 and -3.⁴² mAb 1C7 recognizes the ectodomain of syndecan-3,⁴² and mAb 10H4 is directed against the protein backbone of syndecan-2.⁴³ mAb S1 is directed against the protein core of glypican-1.^{44,45} mAb 3G10 is directed against heparitinase-digested HSPGs⁴⁶ and detects HS regardless of the type of core protein or HS sulfation, only after pretreatment of the sections with heparitinase (heparitinase III, EC 4.2.2.8; Sigma Chemical Co., St. Louis, MO) diluted in 10 mmol/L *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid/2 mmol/L CaCl₂ (pH 7.0) for 1 hour at 37°C. Staining for 3G10 was absent if the sections were not pretreated with heparitinase. The presence of desaturated uronate generated by this enzyme treatment is essential for the reactivity of the antibody. mAb JM403 recognizes an epitope in the HS polysaccharide side chain that contains one or more N-unsubstituted glu-

cosamine units, located in a region with mixed N-sulfated and N-acetylated glucuronic acid-rich disaccharide units.⁴⁷ mAb JM13 is directed against an epitope within the HS polysaccharide side chains containing both N- and O-sulfate groups, in which iduronic acid units are absent (unpublished observations). mAb 10E4 is directed against an N-sulfation-dependent epitope in the HS polysaccharide side chains.⁴⁶

A large number of serial cryosections (5 μ m thick) were used to analyze the expression of various HSPG species in DAT and control brain tissue. At regular intervals sections of each series were stained with anti-A β and anti-tau for comparison with the sections stained for HSPGs. Sections were fixed in acetone for 5 minutes and subsequently in acetone containing 0.15% H₂O₂ for another 5 minutes to block endogenous peroxidase (PO) activity. Subsequently they were preincubated with 20% serum, the type of which was determined by the specific secondary antibody used, for 20 minutes. Sections were incubated overnight at 4°C with primary antibodies followed by incubation with secondary antibodies as indicated in Table 1. Antibodies were diluted in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, which also served as a negative control. Each incubation was followed by extensive washing with PBS. Diaminobenzidine was used as chromogen, and the sections were counterstained with hematoxylin. Finally, the sections were dehydrated with ethanol and xylol.

Immunostaining frequencies (percentage of senile plaques and tangles stained by a specific antibody compared with the anti-A β and anti-tau stainings) were semi-quantitatively and independently scored in any of the following categories 0, 0–50, 50–100, or 100%, by two of the authors (MMV and IO-H). In cases where the scores differed more than one scale unit, consensus was obtained during a re-evaluation.

Congo red staining was performed by consecutive dehydration of acetone-fixed tissue sections with increasing concentrations of ethanol.⁴⁸ After a 20-minute preincubation period in a solution of 3% NaCl in 80% ethanol, sections were incubated for 20 minutes in the same solution containing 0.5% Congo red.

Double Immunostainings

For double immunostaining, sections were fixed and preincubated as described above. Primary antibodies were incubated simultaneously overnight at 4°C, followed by simultaneous incubation with fluorescein-isothiocyanate-conjugated swine anti-rabbit antibodies (Cappel, Boxtel, The Netherlands) and biotinylated horse anti-mouse antibodies (Vector, Burlingame, CA) for 1 hour. Cross-reactivity of these polyclonal antibodies with the primary antibodies was excluded. Finally, sections were incubated with Texas red-conjugated avidin (Vector). To both the fluorescein isothiocyanate- and Texas red-conjugated antibodies, 4% human serum was added to eliminate aspecific binding of the antibodies. All dilutions were made in PBS supplemented with 0.1% bovine serum albumin, which also served as a negative control. Each incubation was followed by extensive washing with PBS.

Enzyme-Linked Immunosorbent Assay

The following mouse proteins (all from Sigma) were coated at 1 μ g/ml and diluted in PBS on a 96-well plate: collagen IV, laminin, and perlecan isolated from the Engelbreth-Holm-Swarm (EHS) tumor, and fibronectin from mouse plasma. Subsequently, wells were incubated with PBS with 1% gelatin and with pAb EY-90 in different dilutions made in PBS supplemented with 0.05% Tween. Wells were then incubated with PO-labeled goat anti-rabbit antibodies diluted 1:2000 in PBS supplemented with 0.05% Tween (Dako, Glostrup, Denmark). Detection was performed with tetramethylbenzidine as chromogen. Positive and negative control reactions for the basement membrane components were included in the experiments. Each incubation was followed by extensive washing with PBS supplemented with 0.05% Tween.

Results

A β , tau, and Ubiquitin in DAT Brains

In all DAT brain tissues, numerous senile plaques were stained with the anti-A β mAb 6C6 (see below). A minority of the senile plaques contained an amyloid core, as identified by Congo red staining. A vast majority of 6C6-positive senile plaques was Congo-red negative, but contained tau-immunoreactive neurites. Two antibodies were used to study tau expression, mAb tau-2 and mAb AT8. Either antibody strongly stained tangles and dystrophic neurites in all DAT brain tissue sections (see below). Neuropil threads were stained more intensely and more frequently by AT8 than by tau-2. The anti-ubiquitin mAb Q1510 stained tangles, dystrophic neurites, and neuropil threads, although the latter were observed at a lower frequency than with AT8 (see below). Neurofibrillary tangles were only occasionally stained by 6C6.

Agrin in DAT Brains

Two well-characterized anti-agrin core protein antibodies, JM-72 and BL-31, were used in this study, and either of these antibody stained similar structures. Agrin was observed in senile plaques in all DAT cases. Virtually all senile plaques, compared with staining with 6C6 (Figure 1a), both in the neocortex and in the hippocampus, were immunopositive for agrin. Generally, staining was prominent and diffusely distributed over the senile plaque area (Figure 1, b and g). In a few cases an additional granular staining was observed (Figure 1, c and d). Small deposits of A β were also stained by JM72 (Figure 1, e and f).

In the hippocampus, neurofibrillary tangles were consistently immunopositive for agrin (Figure 2a). In three DAT cases, so-called "ghost tangles" were identified that were immunoreactive for agrin (Figure 2b), whereas they remained unlabeled with our anti-tau, anti-ubiquitin, or anti-A β antibodies. However, these ghost tangles were labeled by anti-ApoE antibodies (Figure 2c).

In the neocortex, the frequency of staining of neurofibrillary tangles for agrin appeared to be highly variable

Figure 1. Immunohistochemical staining of senile plaques in serial sections (**a-f**) from DAT hippocampus for A β (mAb 6C6) and agrin (mAb JM72). Overview of senile plaques that are diffusely stained for both A β (**a**) and agrin (**b**). Occasionally, an additional granular staining of senile plaques (**c**, mAb 6C6) for agrin (**d**) is observed. Also, small A β deposits (**e**) are immunopositive for agrin (**f**). Higher magnification of a senile plaque stained by JM72 (**g**). Original magnifications: **a-f**: $\times 125$; **g**: $\times 250$.

among the cases studied. In several cases virtually all tangles were positively stained by JM72 or BL31, whereas in other cases only a subpopulation was stained. We did not observe ghost tangles in the neocortex with the anti-agrin or anti-ApoE antibodies. Cerebral blood vessels were consistently strongly labeled by either anti-agrin antibody (Figure 1). The results of the immunohistochemical staining for agrin and other HSPGs in DAT brains are summarized in Table 2.

Perlecan in DAT Brains

Four anti-perlecan core protein antibodies were used in this study: mAbs 1948, 95J10, HK102 and pAb EY-90. In contrast to our observations on agrin, neither senile plaques nor neurofibrillary tangles, both in the hippocampus and in the neocortex, were stained with anti-perlecan mAbs 1948 and 95J10. In contrast, cerebral blood vessels were strongly stained for perlecan with these anti-

Figure 2. Immunohistochemical staining of neurofibrillary tangles in DAT hippocampus for agrin (mAb JM72). Intracellular tangles are stained by JM72 (**a**). Ghost tangles are also stained for agrin (**b**). Cerebral blood vessels are also strongly stained by JM72 (**a** and **b**; examples indicated by **arrowheads**). In **c**, staining of ApoE in ghost tangles (examples indicated by **arrows**) and senile plaques (**arrowheads**) is shown. Original magnification, $\times 125$.

bodies (Figure 3a). With mAb HK102, however, no staining at all was observed in DAT brain sections. Because this antibody was raised against perlecan isolated from the mouse EHS tumor, we performed some additional experiments to certify the specificity of the antibody. Absence of staining with HK102 was observed in both DAT brain cryosections and paraffin-embedded, formalin-fixed DAT brain tissue, the latter either with or without microwave pretreatment. HK102 clearly stained the glomerular basement membrane in mouse kidney cryosections, however, but staining was entirely absent from human kidney cryosections (not shown). Furthermore, HK102 strongly reacted with mouse perlecan purified from the EHS tumor in an enzyme-linked immunosorbent assay (data not shown). These data suggest that HK102 reacts only with mouse perlecan and does not cross-react with human perlecan.

With the anti-perlecan pAb EY-90, a pattern different from that obtained with mAb 1948 or mAb 95J10 was observed. Apart from a consistent strong labeling of the cerebral blood vessels (Figure 3, b and c) this antibody stained coarse granules in senile plaques in both the hippocampus and the neocortex (Figure 3b). The size of the granules varied among the cases studied. These granules clearly differed from dystrophic neurites that were identified by tau or ubiquitin immunostaining (Figure 3, d and e). In 2 of 5 cases, EY-90 stained similar coarse granules in neurofibrillary tangles and in ghost tangles of the hippocampus (Figure 3c), whereas in all cases tangles in the neocortex remained unstained. The specificity of this antibody for basement membrane components was further investigated by enzyme-linked immunosorbent assay. It was found that EY-90 strongly reacts with

mouse perlecan and laminin isolated from the EHS tumor, but only weakly with mouse collagen IV, whereas reactivity with mouse fibronectin was absent, thereby limiting the application of this pAb for the specific detection of perlecan.

Glypican in DAT Brains

One anti-glypican-1 antibody was used in the present study, mAb S1. In senile plaques the expression of glypican-1 was different in the hippocampus as compared with the neocortex. In the hippocampus, only a minority of senile plaques was stained by S1 (less than 50%; Figure 4a), whereas in the neocortex almost all senile plaques were immunopositive. In particular, the amyloid cores of classic senile plaques in the neocortex were stained very intensely (Figure 4b).

Neurofibrillary tangles in both the hippocampus and neocortex were also stained by S1 (Figure 4a). The number of S1-positive tangles, however, varied from only a few to almost all of the tangles that were identified by tau staining. Ghost tangles of the hippocampus were only very weakly stained. Cerebral blood vessels remained unstained by S1.

Syndecans in DAT Brains

The following anti-syndecan mAbs were applied: 10H4 (anti-syndecan-2), 2E9 (anti-syndecan-1 and -3) and 1C7 (anti-syndecan-3). A variable fraction of senile plaques was identified with these antibodies, and, in addition, a regional variation was observed. Of the three antibodies,

Table 2. Summary of Immunohistochemical Staining for HSPGs on DAT Brain Tissue Sections

HSPG species	Hippocampus			Neocortex		Blood vessels [†]
	Senile plaques	Tangles	Ghost tangles	Senile plaques	Tangles	
Agrin	+++	+++	+++	+++	+ / +++	+++
Perlecan [‡]	—	—	—	—	—	+++
Glypican-1	+	+ / ++	— / +	+++	+ / +++	—
Syndecan-2	++	+++	++	+ / +++	— / ++	—
Syndecan-1,-3	+	+++	++	+ / +++	— / +++	—
Syndecan-3	+	+++	+++	+ / +++	+ / +++	+++
HS GAG	+++	+++	+++	+ / +++	+ / +++	+++

The frequency at which the indicated lesions are stained for the HSPG species was assessed by two investigators and analyzed semiquantitatively as follows: +++, all; ++, majority; +, minority; —, none. Senile plaques and tangles were identified by A β and tau staining, respectively.

[†]Blood vessels in both hippocampus and neocortex.

[‡]Based on staining patterns of mAbs 1948 and 95J10.

Figure 3. Immunohistochemical staining of DAT hippocampus with anti-perlecan antibodies. Cerebral blood vessels are stained by mAb 95J10 (a). The anti-perlecan pAb EY-90 stains coarse granules in both senile plaques (b) and neurofibrillary tangles (arrows) in DAT hippocampus (c). Blood vessels are also strongly stained by EY-90. These coarse granules in senile plaques remain distinct from dystrophic neurites that are stained for tau (d) and ubiquitin (e). Many scattered neuropil threads are detected with anti-tau and only a few by anti-ubiquitin. Original magnification, $\times 100$.

10H4 stained a majority of senile plaques in the hippocampus, whereas a smaller number, approximately 50%, of the senile plaques were stained with either 2E9 or 1C7 (Figure 5a, d, and g). In contrast, in the neocortex all three antibodies stained a comparable number of senile plaques that composed between 25 and 100% of the senile plaques identified with mAb 6C6. Similar to S1, mAb 10H4 particularly stained the amyloid cores of classic senile plaques very intensely.

All three antibodies also stained neurofibrillary tangles. In the hippocampus, virtually all tangles were stained with all three antibodies (Figure 5, b, e, and h). Ghost tangles in the hippocampus were also labeled by the three antibodies (Figure 5, c and f). In the neocortex, however, a highly variable number of tangles were stained by these three antibodies. The number of tangles stained by 1C7, 2E9, or 10H4 varied from only a few to virtually all tangles identified by tau immunostaining. Only in a few cases was staining for tangles completely absent with 2E9 or 10H4.

Blood vessels were not stained by 10H4 or 2E9. In contrast, 1C7 faintly stained capillaries and small arterioles and strongly stained larger vessels with a pattern reminiscent of intercellular junctions between both endothelial cells and smooth muscle cells (not shown). 1C7 also clearly stained reactive astrocytes, either clustered around amyloid-containing senile plaques or scattered throughout the section (Figure 5i).

HS GAGs in DAT Brains

Expression of HS GAG side chains was studied by using four different antibodies: 3G10, JM403, JM13, and 10E4. In contrast to the other 3 mAbs, 3G10 reacts only with the stub of heparitinase-digested GAG side chains. Numerous senile plaques, both in the hippocampus and in the neocortex, were stained with all of these antibodies (Figure 6, a, d, e, f, and i).

In the hippocampus both intracellular and ghost tangles were all labeled by any of these four anti-HS antibodies (Figure 6, a-c and g-i). Strongest staining was observed with 3G10. Figure 7 shows an example of 3G10 staining of both tau-positive intracellular tangles and tau-negative ghost tangles. In the neocortex, a variable number of tangles, ranging from only a few to virtually all tangles present, were stained by the anti-HS antibodies. Only occasionally was staining absent. Blood vessels were always stained for HS GAGs. In addition to these staining patterns, mAb JM13 uniformly stained the white matter of all brain sections in a fine granular way (Figure 6f).

Control Brains

Diffuse senile plaques, without associated tau or ubiquitin immunoreactivity, were identified at a low frequency with

Figure 4. Immunohistochemical expression of glypican-1 with mAb S1 in DAT hippocampus (a) and cortex (b). S1 stains senile plaques and tangles (a). In the cortex the cores of amyloid plaques are accentuated by S1 (b). Original magnification, $\times 100$.

Figure 5. Immunohistochemical staining of syndecans in DAT brain tissue. Shown are staining of senile plaques (**a**), tangles (**b**), and ghost tangles (**c**) by the anti-syndecan-2 mAb 10H4; staining of senile plaques (**d**), tangles (**e**), and ghost tangles (**f**) by the anti-syndecan-3 mAb 1C7; and staining of senile plaques (**g**) and tangles (**h**) by the anti-syndecan-1 and -3 mAb 2E9. mAb 1C7 also stains reactive astrocytes associated with senile plaques (**i**). In **b** and **g**, tissue sections from DAT neocortex are shown; all others are hippocampal sections. Original magnification, $\times 100$.

mAb 6C6 in control brains (Figure 8a). Only very incidentally were dystrophic neurites and/or neurofibrillary tangles identified with anti-tau or anti-ubiquitin antibodies in control brain tissue. Staining of senile plaques, tangles, and blood vessels with the above-mentioned HSPG antibodies in control brain tissue was similar to that observed in DAT brain tissue. An example of agrin staining (mAb JM72) in diffuse senile plaques of control brains is shown in Figure 8b.

Discussion

Agrin is a recently cloned basement membrane-associated HSPG with a core protein of 212 kd.^{32–34} It induces the clustering of acetylcholine receptors during the development of the neuromuscular junction, the activity of which is dependent on the different isoforms that are produced by alternative splicing.^{49–51} This alternative splicing of agrin involves the insertion of small oligopeptides at least at three defined sites within the molecule,⁴⁹ resulting in x-, y- and z-agrin. In this study we demonstrated that agrin is associated with both senile plaques and neurofibrillary tangles of the hippocampus and the neocortex. Only in a few DAT cases was agrin expression in cortical tangles lacking. Moreover, we demonstrated the association of the HSPGs glypican and syndecan

with senile plaques and tangles, although at a lower frequency than agrin, and the absence of such an association of the HSPG perlecan with these lesions. Furthermore, we demonstrated the expression of HS GAG side chains in senile plaques and in neurofibrillary tangles, confirming previous reports.^{9,11,17,36} Of all HSPG species studied, agrin appeared to be most consistently and uniformly associated with senile plaques and tangles, confirming a recent study.⁵² So far, however, we have not been able to make a distinction between x-, y- and z-agrin.

HSPGs are an invariant component of senile plaques, tangles, and systemic human amyloids. One of the possible functions ascribed to HSPGs is the stabilization of the amyloid by providing protection against proteolytic degradation and removal.⁷ In this respect, agrin may play an important role as stabilizer of A β and tau deposits because it contains nine follistatin-like protease inhibitor domains.³⁴ This putative function of agrin may be illustrated by the way ghost tangles are thought to be formed. It is generally accepted that ghost tangles originate from intracellular tangles. When neurons die, tangles remain as extracellular or ghost tangles that have become inert structures and that have lost tau and ubiquitin reactivity^{53,54} but are ApoE positive.⁵⁵ Our observation that agrin is found in both intracellular and ghost tangles may sup-

Figure 6. Immunohistochemical staining of HS GAG side chains in DAT brain hippocampus (**a-e, g, and h**) and neocortex (**f and i**). The anti-HS stub mAb 3G10 stains senile plaques (**a**), tangles (**a and b**), and ghost tangles (**c**). HS GAG side chains are identified in senile plaques by the mAbs JM403 (**d**), 10E4 (**e**), and JM13 (**f and i**). HS GAG side-chain expression in tangles is demonstrated by staining with JM403 (**g**), 10E4 (**h**), and JM13 (**i**). In **f**, a uniform staining of white matter by JM 13 is shown in addition to staining of senile plaques. All four anti-HS GAG antibodies label cerebral blood vessels. Original magnification, $\times 100$.

port the idea that agrin acts as a protease inhibitor, providing resistance of tangles—and possibly also of senile plaques—to degradation by extracellular proteases. Because it has been described that agrin is produced by neurons⁵⁶ and that it may also be involved in the maintenance of synapses between neurons within the central nervous system,⁵⁷ it can be speculated that degeneration of neurons in DAT brains leads to an altered distribution of neuronal agrin and its subsequent association with neurofibrillary tangles. Possibly, the agrin found in senile plaques has a neuronal origin as well. The exact

cellular source of agrin remains to be investigated however. On the contrary, perlecan is not associated with ghost tangles and does not contain protease inhibitor domains. HS GAG side chains may act as a co-factor for the activation of serine protease inhibitors by binding to these protease inhibitors, as is well known for the activation of antithrombin III by heparin binding.⁵⁸

We were unable to reproduce the findings of Snow et al,^{10,17} who observed staining of senile plaques and tangles for perlecan with mAb HK-102. In our hands HK102 stained neither cerebral blood vessels nor senile plaques

Figure 7. Double immunofluorescence staining for HS GAG (mAb 3G10) and tau of an intracellular tangle and a ghost tangle. Both the intracellular tangle and the ghost tangle are stained by 3G10 (**a**), whereas only the intracellular tangle is stained by the anti-tau antibodies (**b**). Original magnification, $\times 1000$.

Figure 8. Immunohistochemical staining of senile plaques in serial sections from control hippocampus. Diffuse senile plaques in control brain tissue are stained for both A β (a, mAb 6C6) and agrin (b, mAb JM72). Original magnification, $\times 125$.

and tangles. Two other anti-perlecan mAbs, 1948 and 95J10, stained neither senile plaques nor tangles, whereas the basement membrane of cerebral blood vessels was strongly stained, indicating that our immunohistochemistry techniques are not limited by lack of sensitivity. These data are largely in accordance with the findings of van Gool et al,³⁶ who observed a strong staining of blood vessels for perlecan and only a very weak staining of neuritic senile plaques, but no staining of other plaque types or tangles. In this study, formalin-fixed paraffin-embedded tissue was used, however, which does not allow for an exact comparison with our results. Furthermore, we found that mAb HK-102 reacted only with mouse perlecan, and not with human perlecan in murine and human renal tissue, respectively, which explains the lack of immunoreactivity in DAT brains. It was also noted by Kato et al,⁴⁰ who produced and characterized the mAb HK-102, and confirmed by the commercial supplier that this antibody reacts only with mouse antigens. With the polyclonal anti-perlecan antibody (EY-90), we observed a different staining pattern however. The reactivity of this pAb is less defined than that of the above-mentioned mAbs, because this antibody has been raised against a crude HSPG fraction from the mouse EHS tumor, which contains—among other components—mouse perlecan. In DAT brains, EY-90 stained coarse granules, different from dystrophic neurites, in senile plaques of both the hippocampus and neocortex and in tangles of the hippocampus. Staining of similar coarse granules around congophilic plaque cores with antibodies to laminin and collagen IV has been described.⁵⁹ Decoration of coarse deposits with anti-HSPG antibodies around amyloid-laden vessels has also been demonstrated in brains of patients with hereditary cerebral hemorrhage with amyloidosis of the Dutch type, but these deposits were not spatially associated with senile plaques.⁶⁰ The discrepancy between the results that we obtained with mAbs 1948 and 95J10 on the one hand and with the pAb EY-90 on the other can be explained by the cross-reactivity of the latter antibody with laminin in senile plaques, as we observed by enzyme-linked immunosorbent assay, resulting in staining of the granules, whereas the vascular staining obtained with this antibody could be interpreted as staining of both perlecan and laminin.

We showed that in DAT brains perlecan is expressed exclusively in the basement membrane of blood vessels and neither in senile plaques nor in tangles. We postulate that perlecan plays only a minor role in DAT pathogenesis, despite the fact that perlecan has been shown *in vitro*^{18–20} and in a rat model²² to bind to A β and accelerate A β fibril formation. However, these activities are at least in part mediated by the HS GAG side chains of perlecan. All HSPGs, including agrin, glypican, syndecan, and perlecan, contain HS GAG side chains composed of similar sugar units, but ones that may differ in the degree of N- and O-sulfation, acetylation, and percentage of iduronic acids in the chain. Therefore, agrin may use the same activity as perlecan in binding A β and accelerating A β fibril formation through its HS GAG side chains, but this activity may also depend on the degree of modification of the HS GAG side chain. Furthermore, agrin may participate in tangle formation, because it has been demonstrated that sulfated GAGs stimulate the phosphorylation of tau and inhibit the binding of tau to microtubules, thus promoting the formation of paired helical filaments.^{27,28,31} It remains to be elucidated, however, whether agrin indeed displays these activities and how it becomes attached to the tangles.

The results of the present study show that members of the syndecan and glypican family of HSPGs are associated with senile plaques and tangles, albeit to a lesser extent than agrin, because the proportion of DAT lesions stained for these factors is smaller and the expression is more dependent on the specific region in the brain. It has been reported previously that, using the same antibodies, syndecans and glypican-1 were not expressed in tangles and only to a minor extent in senile plaques.³⁶ This discrepancy is likely due to the use of formalin-fixed, paraffin-embedded brain tissue in the latter study. Many epitopes may have been destroyed during the tissue preparation, whereas they remain intact and available for antibody recognition in unfixed cryosections.

The expression of HSPGs is dependent on the specific region in the brain as we observed for the hippocampus and neocortex. In general, reactivity of HSPG antibodies was more frequently observed in tangles of the hippocampus than in those of the neocortex. A number of studies have shown that there is a regional variability in the expression of several A β -associated factors in DAT

brains. For example, intercellular adhesion molecule-1 is expressed in senile plaques of the neocortex and hippocampus, but not in cerebellar senile plaques,⁴⁸ whereas ApoE is found in senile plaques of the neocortex, hippocampus, and cerebellum, but not in the striatum.⁶¹ Future studies may reveal whether the HSPG species studied in this paper are also differentially expressed in the various regions of the DAT brain, which may shed more light on their role in the formation of senile plaques and tangles and on the cellular source of these HSPGs.

In conclusion, of the HSPG species studied, agrin is the most consistently detectable type of HSPG in senile plaques and tangles of DAT brains and may, via its protease-inhibitor domains of the protein backbone, protect aggregates of A β in senile plaques and of tau in tangles from extracellular proteolytic degradation. In addition to agrin, glypican and syndecans, but likely not perlecan, may—via their HS GAG side chains—contribute to the formation of senile plaques and tangles in DAT brains. The exact role of these HSPGs remains to be elucidated however.

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